REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE May 28 2003	3. REPORT TYPE AND DATES COVERED Final: 01 Feb 01 -- 31 Jan 03

4. TITLE AND SUBTITLE
Engineered plants as biosensors

5. FUNDING NUMBERS

DAAD19-01-1-0023

6. AUTHOR(S)

Neal Stewart

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UNCG, 100 McIver, Greensboro, NC 27403

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

42300.1-LS

11. SUPPLEMENTARY NOTES

12 a. DISTRIBUTION / AVAILABILITY STATEMENT

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

The aim of the research was the creation of a model biosensing plant that could detect plant diseases and to characterize the utility of laser induced fluorescence imaging for detecting the inducible (LIFI) plant signal. Tobacco was engineered with a plant pathogen inducible promoter (GN1) fused to a green fluorescent protein (GFP). GFP was upregulated in response to a salicylic acid simulant (BTH) and plant pathogens (PART 1). GFP fluorescence was low, but detectable. LIFI I/O was characterized for highly fluorescing transgenic plants (PART 2).

14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT **UNCLASSIFIED**	18. SECURITY CLASSIFICATION ON THIS PAGE **UNCLASSIFIED**	19. SECURITY CLASSIFICATION OF ABSTRACT **UNCLASSIFIED**	20. LIMITATION OF ABSTRACT **UL**

Part 1.

Pathogen inducible reporting in transgenic tobacco using a GFP construct

Preprint—Plant Sciences

Mitra Kooshki[1], Mentewab Ayalew[2] and C. Neal Stewart, Jr.[2]*

1. Current address:

Wake Forest University School of Medicine

Dept. Radiation Oncology

Radiation Biology section

4 th floor, NRC bldg.

Winston Salem, NC 27157

2. Current address:

The University of Tennessee

Department of Plant Sciences and Landscape Systems

Rm 252 Ellington Plant Sciences

2431 Center Drive

Knoxville, TN 37996-4561 USA

* Corresponding author

Summary

A model sentinel plant (phytosensor) capable of signaling pathogen attack in the field was obtained by transformation with a construct containing the green fluorescent protein (GFP) as a reporter gene, driven by a tobacco glucanase promoter. The *gn1* promoter is responsive to salicylic acid, which is synthesized by the plant during systemic acquired resistance (SAR). This is a first step towards the creation of early disease diagnostics using sentinel plants with an integral real-time, fluorescent-based reporting mechanism. Transgenic plants were sprayed with benzothiadiazole (BTH), a salicylic acid analogue, at different stages of development. The presence of GFP transcript in transgenic plants after induction with 5 mM BTH was determined. GFP was detected as early as 48 hours after induction using western blot analysis, but the fluorescence could not be robustly detected spectrally. Plants younger than 8 weeks did not express detectable levels of GFP. Gn1/GFP was systemically induced by BTH in treated and nontreated plant tissues. Time course of induction of the *mgfp5-er* gene by BTH in transgenic plants showed that induction and GFP accumulation was slow and maximum GFP accumulation was found between 6-12 days after treatment. *Gn1/gfp* expression was also induced by inoculating plant leaves with the pathogen *Plectosporium tabacinum*.

Introduction

Crop terrorism countermeasures and precision agriculture share at least one common need: the ability to sense plant pathogens before they cause widespread damage. All of the current early warning detection technologies rely on field-level monitoring by visual observation of disease symptoms. In recent years, several groups have experimented with remote sensing of plant disease using hyperspectral spectrometry. These approaches suffer from detecting plant disease post-symptomatically. The goal of our research is to produce plant biosensors (or phytosensors) that can be used as early-warning sentinels of disease. It is feasible to fuse the reporter gene coding for green fluorescent protein (GFP) to pathogen inducible promoters to produce disease-reporting organisms. The use of GFP as a reporter gene for phytosensors has several advantages over commonly used reporter genes, such as firefly luciferase [1] and GUS [2]. It is non-destructive, it does not need any substrate or cofactors, it can report in real-time and can be remotely sensed. Thus GFP has become a useful reporter marker for gene expression and regulation in plants [3]. Furthermore the *mgfp5-er* variant is optimized for expression in plants [4] and, in our hands, proven to be the optimal whole plant visible trangenic fluorescent marker in the field [3,5].

Plants have several defense mechanisms that enable them to respond to pathogen attack. Two forms of response mechanisms are local defense and systemic acquired resistance (SAR). The localized defense, which is termed hypersensitive response (HR) [6] occurs at the site of attack and results in restricted lesions clearly delimited from surrounding healthy tissues.. SAR results in broad-spectrum, long-lasting immunity in

non-infected tissues, which provides protection not only against the inducing pathogen but also against a spectrum of pathogens including viruses, bacteria, and fungi [7]. A number of experiments have been performed to study the pathogen inducibility of several defense-related genes. One example is the *pr2* gene, which encodes for a glucanase and is expressed during HR in tobacco leaves inoculated by tobacco mosaic virus (TMV) [8]. Another is *pr1*, characterized in tobacco and *Arabidopsis*, which is a highly abundant and tightly regulated gene that is expressed during SAR [9]. The *Nicotiana plumbaginifolia gn1* gene, encoding a ß-1,3-glucanase isoform, has also been characterized. *gn1* was found to be expressed in roots and older leaves and is controlled by a pathogen-inducible promoter. The expression pattern suggested that the characterized ß-glucanase played a role in plant development and in the defense against pathogen infection [10].

Salicylic acid (SA) has been shown to play a central role as a signaling molecule involved in both local defense reactions and in the induction of SAR [11]. The level of SA increases after pathogen inoculation and it is correlated with SAR [12]. Moreover, exogenous application can induce SAR [9]. Transgenic tobacco and *Arabidopsis thaliana* plants expressing the bacterial *nahG* gene encoding salicylate hydrogenase, an enzyme that catalyzes the conversation of SA to catechol, not only produce low levels of SA, but are unable to express SA in response to viral, fungal or bacterial pathogens [13,14]. These results suggest that SA is required in the SAR signal transduction pathway.

SA regulates many pathogenesis related genes including *pr1* [15], *pr2* [16], *pr4* [17], and *pr5* [18]. The effect of SA on the *gn1* promoter was evaluated before and after spraying transgenic tobacco plants with 5 mM SA. SA treatment induced the *ggn1* promoter about 14-fold, based on increased GUS activity [10]. Analysis of GUS activity

in transgenic tobacco plants containing the *gn1/gus* construct showed different GUS activity levels in different tissues.. The maximum GUS activity was observed in older leaves and roots. No detectable activity was present in the upper parts of the transgenic plants. Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) is another inducer of SAR in a number of plants including wheat, *Arabidopsis*, and tobacco [19-21]. BTH mimics SA in its induction of SAR genes [20]. Narusaka et al. [22] showed that BTH induces the expression of chitinase gene and disease resistance both locally and systemically. Systemic disease resistance appeared 24 h after the treatment of cucumber plants with 0.5 mM BTH.An agrichemicals company, Syngenta, markets BTH as a crop protectant under the brand name " Actigard ".

To study the feasibility of utilizing pathogen-reporting phytosensors the *gn1* promoter was fused to an *mgfp5-er* reporter that is targeted to the ER, and transgenic plants were produced. The goal was to determine the patterns of GFP-mediated fluorescent signaling post-BTH application and/or pathogen infection.

Methods

Construction of *gn1/gfp* vector and plant transformation

The plasmid containing the *gn1* promoter was kindly provided by Marc van Montagu, Gent, Belgium [10]. A 1.6 Kb fragment containing the *gn1* promoter was PCR amplified to introduce SacI restriction sites at both ends. Primers used for amplification were forward 5' CGGCAGAGCTCGTAATCACGT 3' and reverse 5' GGCGGGAGCTCGGCTTTTGTT 3'. The PCR product was cloned into a pBinplusARS derivative plasmid (kindly donated by Bill Belknap) containing the *mgfp5-er* gene, a nos

terminator and a kanamycin selection cassette. The orientation of the *gn1* fragment was confirmed by digestion with PacI/KpnI and the construct was partially sequenced to ensure the ATG initiation codon was in frame with the *gfp* gene. The resulting plasmid, called pBinplus Gn1, had a molecular size of 14.5 Kb.

Nicotiana tabacum cv. Xanthi seeds were sterilized by soaking in 20% Clorox for 5 minutes and in 70% alcohol for 2.5 minutes. The seeds were then washed with sterile water and grown in sterile conditions on MS basal medium [23] for 4 weeks. The plasmid pBinplus Gn1 was transformed into *Agrobacterium tumefaciens* strain GV3850 and tobacco was transformed by leaf disc transformation method [24].

Transgenic plants

Primary transgenic plants were grown in a growth chamber under 300 μE irradiance, 16 hr photoperiod at 20° C. The plants were then transferred to the greenhouse and the seeds were collected after 3 months. Seeds from T_0 transgenic tobacco plants were surface sterilized and grown in Petri dishes containing MS medium supplemented with 200 μg/ml kanamycin. After 4-8 weeks the plants were transferred to MS media containing 3 mM BTH. Protein was extracted from the leaves and western blot analysis was performed as described by Stewart et al. [25].

Preparation of conidia from *Plectosporium tabacinum* and inoculation of tobacco leaves

Plectosporium tabacinum (Van Beyma) [26] strain MEP 1353 obtained from the American Type Culture Collection (ATCC) were grown on media containing potato

dextrose agar (PDA) at 24º C for 2 weeks and conidia was harvested from the plates using 5 ml water. T_1 tobacco leaves were inoculated by placing 10 µl of 3x 10^7 conidia /ml on the leaves and the plants were incubated at 22º C in the growth chamber under cover for 48 hours.

DNA and RNA analysis

DNA extraction from plant tissue (CTAB method) and PCR were performed as described by Stewart et al. [27]. RNA extractions were done from tissue samples taken 7 days after BTH treatment of twelve-week-old plants . Leaf tissue was ground to a powder in liquid nitrogen and total RNA was extracted using RNA extraction columns according to the manufacturer's protocol (Qiagen).

RT-PCR analysis of transformants

RNA was reverse transcribed using the SuperScript^{TM} First-Strand Synthesis System for RT-PCR according to the manufacturer's protocol (Invitrogen). PCR was performed on cDNA by using forward primer F1 5'AAAAGAGCTCATCCAAGGAGATATAACAAT 3' and reverse primer R1 5' CCGGTTGAGCTCTTAAAGCTCATCATGTTT 3' to estimate the transcript levels of the *mgfp5-er* transgene in the treated and untreated plants.

Fluorescence spectroscopy

To determine the expression of GFP in response to BTH treatment, GFP activity was analyzed by fluorescence spectrophotometry in leaves of transgenic plants before

and after BTH treatment. The detection of GFP florescence on intact tissues was attempted using a Fluoromax-2 fluorescence spectrometer (Jobin Yvon, Instruments S.A. Inc., Edison, NJ). The treated and untreated leaves were excited at 385 nm, and emission spectra were recorded from 400 to 550 nm according to published methods [28].

Results

Gn1/gfp vector and transgenic plants

For the proof-of-principle of the phytosensor concept, a chimeric *gn1/gfp* reporter gene was constructed, and introduced into tobacco and plants were analyzed.

Forty plants resistant to kanamycin were chosen for regeneration. These plants as well as a subsample of their progenies were tested for the presence of the *mgfp5-er* gene by PCR analysis. All putative transgenic primary plants were PCR positive (data not shown). Progeny analysis was performed to estimate the number of T-DNA loci in 20 transgenic tobacco events by analyzing the segregation of the kanamycin resistant and sensitive phenotype. Of these, 75% showed ratios of 3 kan^r: 1 kan^s (Chi-squared analysis, data not shown). These results indicated which events had single T-DNA locus inserts per genome. T$_1$ progeny from this subsample of single-locus events were used for subsequent analyses.

RNA analysis of transgenic plants induced with BTH

Twelve-week-old primary transgenic plants that were grown on MS media containing kanamycin were sprayed with 5 mM BTH. Leaf samples were collected 7 days after induction. RT-PCR was performed to determine the presence of GFP

transcripts and identify high-induction events. The variation of transcript levels was large among events with all events expressing GFP to some degree (Fig. 1), albeit at greatly reduced levels compared with the transgene under the control of the constitutive 35S promoter [5].

BTH-induced expression of a chimeric *gn1/gfp* gene in T$_1$ transgenic tobacco plants

Four- and eight-week-old transgenic progeny transgenic plants resistant to kanamycin grown in Magenta boxes on MS medium and 3 mM BTH were used to characterize the induction dynamics of GFP synthesis in response to BTH. No *gfp* expression was detected using western blot analysis in small and young tobacco plants (Fig. 2A). In older plants GFP was not detected after 48, 72, and 96 h post induction, but was detectable nine days after BTH application. At days 11 and 18 GFP expression was detected in some of the transgenic plants treated with 3 mM BTH. Using this system, GFP expression was low and difficult to detect using western blot analysis (Fig. 2B). In another set of experiments, twelve-week-old plants grown in soil were sprayed with 5 mM BTH. Western blot analysis was performed on transgenic plants 48 h after induction, showing expression of GFP in most treated lines but not in water-sprayed transgenic plants. (Fig. 2C).

GFP detection in transgenic plants expressing the *gn1/gfp* construct by fluorescence spectrophotometry and UV light

No constitutive expression of GFP was observed by fluorescence spectrophotometry or UV light before treatment with BTH. One week after spraying the

selected transgenic plants with 5 mM BTH, the lower leaves and roots were screened for *gfp* expression. GFP fluorescence was detectable in a single 12 week-old plant in the roots and leaves and *35s/mgfp5-er* plants (positive control) (Figs. 3 and 4), however the fluorescence was very low and was not detectable by fluorescence spectrophotometry.

Systemic induction

Previous studies have shown that treatment of the lower leaves with BTH in cucumber plants, brought about the accumulation of chitinase, another PR protein, in the untreated, upper leaves, as well as in the primary treated leaves [29]. To investigate the systemic induction of the *gn1/gfp* by BTH, GFP activity was by spraying with both BTH and water. The 4th leaf was sprayed with water whereas the rest of the plant was sprayed with BTH.

We found that external application of BTH to the transgenic tobacco plants harboring the *gn1/gfp* construct led to GFP expression in treated and untreated leaves of the same plant (Fig. 5). The result showed that the BTH treatment could induce systemic expression of the *gn1/gfp* gene.

Time course of induction of *gn1/gfp* gene by BTH in transgenic tobacco plants

Western blot analysis was performed on forty transgenic events to determine the levels of GFP expression. Out of those 19 lines expressed GFP at detectable levels after BTH treatment. Four events with high GFP expression were used for the time course analysis. These independent transgenic events (events # 3, 6, 8, 11) had strong signals in western blots at different days after treatment with BTH. Gn1/GFP. Plants were treated

with water or BTH (5 mM) and incubated in a growth chamber. Leaf samples were collected at various periods extending to 16 days. Western blot analysis of BTH-treated transgenic plants demonstrated that GFP could be detected 48 hours after treatment. Levels of GFP protein gradually increased to a maximum at one week and began to decrease at day 16 (Fig. 6). GFP accumulation was slow and the maximum was reached between 6-12 days (Fig. 6).

Fungal inoculation

Twelve-week-old transgenic tobacco plants (T_1) were inoculated with spores of the tobacco fungal pathogen *Plectosporium tabacinum.* Eight transgenic events were used for this experiment. A hypersensitive response was detected on the leaves and stems 10 days after inoculation. Samples from the lower leaves, close to the inoculated area were collected and western blot analysis was performed. GFP expression was detected by western blot analysis upon pathogen treatment (Fig. 7).

Discussion

The induction of genes encoding enzymes such as chitinase, glucanase, and peroxidase occur in different plant species in response to pathogen infection, and application of certain organic and inorganic compounds. The relationship between glucanase and disease resistance in tobacco has been investigated [30,31].

It has previously been reported that SAR is induced in response to treatment with BTH and SA in plants [21, 29]. Treating the first (lowest) leaf with these compounds induces a systemic protection in the second and third leaves without causing any stress signs or damages.

Previous studies have been conducted on systemic induction of PR protein (chitinase) by using BTH and SA as inducers. Systemic resistance could be induced in leaves treated with BTH and transferred to the untreated upper leaves. In contrast, the application of SA does not induce systemic signal and apparently SA induced local resistance. Also, another study showed that BTH induced SAR in wheat [19] against powdery mildew infection by activation of pathogenesis-related gene and the genes associated with SAR. In our experiments, we found that regulation of the *gn1* promoter and expression of *gfp* after treatment with BTH is systemic and detectable in untreated leaves as much as in treated lower leaves. These results are encouraging when contemplating the need to measure GFP fluorescence in accessible plant organs.

Previous studies have shown that transgenic tobacco plants containing *gn1/gus* expressed high GUS activity in roots compared to the upper parts of plants [10]. In this regard, we screened the roots of independent transgenic events for GFP fluorescence after treatment with BTH. Low amounts of GFP fluorescence was detectable in the lower leaves and especially in the roots of one transgenic plant compared to negative and positive control plants (Figs. 3 and 4). Fluorescence was very low to begin with and was not detectable by fluorescence spectrophotometry. As the result of relatively less autofluorescence we were able to detect low levels of fluorescence in roots by visual inspection in the dark upon illumination by UV light. In contrast to GFP fluorescence in shoots, fluorescence in roots would not be very accessible for field-based monitoring of sentinel plants-- phytosensors.

Although GFP was detectable by western blot analysis in most of transgenic events, we were unable to detect strong GFP fluorescence in transgenic plants after

induction with BTH. High-level background fluorescence and low-level *gfp* expression in plant tissues could be the main reasons that GFP fluorescence could not be robustly optically observed. Comparing GFP and GUS as reporter markers showed that GFP might under-report promoter activity in some transgenic plants [31]. For instance, *gfp* expression driven by *Arabidopsis thaliana* zinc finger protein (AtZFP) promoter is difficult to distinguish from background autofluorescence present in many *Arabidopsis* tissues [32]. In our experiments, *gfp* expression driven by the inducible promoter, *gn1*, could not be detected by fluorescence spectrophotometry.

Tobacco tissues were challenged with an incompatible fungal pathogen, *Plectosporium tabacinum*. The results of fungal inoculation were the induction of a pathogenesis related gene and the expression of a *gfp* gene. Transgenic plants showed a similar pattern of expression when treated with BTH or inoculated with *Plectosporium tabacinum*, indicating this kind of inducibility may be part of the basis for broad-spectrum immunity of plants to most pathogens. There is a great number of PR proteins in plants and their functions and gene expression may not be the same in all species. Our experimental data obtained from tobacco may be useful information for understanding of the function of glucanase and its expression.

Previous reports have confirmed that transgenic tobacco plants containing 5' non-coding region of a *gn1/gus* reporter gene was found to be expressed in roots and older leaves [10]. GUS expression was induced in response to plant stress treatments such as SA, ethylene, wounding, and bacterial infection. Treatment with two different bacterial species, *Pseudomonas syringae*, and *Erwinia carotovora* resulted in hypersensitive reactions and showed high level of GUS activity in transgenic plants. When the tissue

extracts from BTH-induced leaves of pBinplus Gn1 transformants were analyzed for

GFP, the detection level appeared to be lower than that reported in SA-induced tobacco

plants containing the *gn1/gus* construct [10]. Using more advanced imaging techniques or

enhancing *gfp* expression may overcome these problems. Clearly, the low GFP

fluorescence in *gn1/gfp* transgenic plants is an impediment to the realization of deploying

a field-level, transgenic phytosensor that could report pathogen infection in real time.

One possible tool to increase expression, and hence, utility, is to add an enhancer element

flanking the promoter to boost the promoter. The 35S promoter of cauliflower mosaic

virus contains complex enhancer elements that can activate or affect gene expression

when present upstream or down stream of genes [32, 15]. The 5'-upstream region (-90 to

-419) of CaMV 35S promoter could be used in the form of tandem repeats to enhance

gene expression. We will then be left with the problem of decreasing the time required

between induction and detectable expression (fluorescence) to be evident. Seven days

post-infection phytodetection is too long to be of practical use. Still, if expression could

be significantly enhanced, a 3-4 day post-infection time-to-fluorescence is reasonable

(see Fig. 6). Ultimately, the scheme for a real-time phytosensor with a fluorescent output

may depend on the utilization of PR-type promoters with enhancers coupled with

synthetic promoter motifs. The desired outcome will be induction to a wide range of

pathogens, high expression and little lag. The application of such phytosensors may

someday prove to be an important tool early detection of routine agricultural plant

diseases as well as agroterrorism activity.

Acknowledgements.

BIBLIOGRAPHY

[1] D. Ow, K. Wood, D.A. Deluca, J.R. De Wet, D.R. Helinski, S. Howell, Transgenic and stable expression of the firefly luciferase gene in plant cells and transgenic plants. Science 234 (1986) 856-859.

[2] R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, Gus fusion; ß- glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6 (1987) 3901-3907.

[3] C.N. Stewart, Jr., The utility of green fluorescent protein in transgenic plants. Plant Cell Rep. 20 (2001) 376-382.

[4] J. Haseloff, K.R. Siemering, D.C. Prasher, S. Hodge, Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Sci. USA 94 (1997) 2122-2127.

[5] B. K. Harper, S.A. Mabon, S.M. Leffel, M.D. Halfhill, H.A. Richards, K.A. Moyer, C.N. Stewart, Jr. Green fluorescent protein in transgenic plants indicates the presence and expression of a second gene. Nature Biotech. 17 (1999) 1125-1129.

[6] K.E. Hammond-Kosack, J.D.G. Jones, Resistance gene-dependent plant defense responses. Plant Cell 8 (1996) 1773-1791.

[7] J. Ryals, K. Lawton, T. Delaney, L. Friedrich, H. Kessmann, U. Neuenschwander, S. Uknes, B. Vernooij, K. Weymann, Signal transduction in systemic acquired resistance. Proc. Nat. Acad. Sci. USA. 92 (1995) 4202-4205.

[8] F. Cote, J. R. Cult, A. Asselin, D. F. Klessing, Pathogenesis related acidic ß- 1,3-glucanase genes of tobacco are regulated by both stress and developmental signals. Mol. Plant Microbe Interact. 4 (1991) 173-181.

[9] E. R. Ward, S.J, Uknes, S.C.Williams, S.S. Dincher, D. L. Wiederhold, D.C. Alexander, P. Ahl-Goy, J.-P. Métraux, J. A. Ryals, Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3 (1991) 1085-1094.

[10] C. Castresana, F. de Carvalho, G. Gheysen, M. Habets, D. Inzé, M. Van Montagu, Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* ß -1,3- glucanase gene. Plant Cell 2 (1990) 1131-1144.

[11] J. Durner, J. Shah, D.F. Klessig, Salicylic acid and disease resistance in plants. Trends Plant Sci. 2 (1997) 266-274.

[12] J. Malamy, J. P. Carr, D.F. Klessig, I. Raskin, Salicylic acid: a likely endogenous signal in the resistance of tobacco to viral infection. Science 250 (1990) 1002-1004.

[13] T.P. Delaney, S. Uknes, B.Vernooij, L. Friedrich, K. Weyman, D. Negrotto, T. Gaffney, M. Gutreila, H. Kessmann, E. Ward, J. Ryals, A central role of salicylic acid in plant disease resistance. Science 266 (1994) 1247-1250.

[14] T. Gaffney, L. Friedrich, B. Vernooij, D. Negrotto, G. Nye, S. Uknes, E. Ward, H. Kessmann, J. Ryals, Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261 (1993) 754-756.

[15] E. Kombrik, I. E. Somssich, Pathogenesis related proteins and plant defense. Mycota 5 (1997) 107-128.

[16] J. Shah, D.F. Klessig, Identification of a salicylic acid responsive element in the promoter of the tobacco pathogenesis related ß-1,3 -glucanase gene, PR2d. Plant J. 10 (1996) 1089-1101.

[17] S. Potter, S. Uknes, K. Lawton, A. M. Winter, D. Chandler, J. Dimaio, R. Noviyzky, E. Ward, J. Ryals, Regulation of a hevein like gene in *Arabidopsis.* Mole. Plant Microbe Interact. 6 (1993) 680-685.

[18] P. Epple, K. Apel, H. Bohlmann, An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis related proteins. Plant Physiol. 109 (1995) 813-820.

[19] J. Görlach, S. Volrath, G. Knauf-Beiter, G. Hengy, U. Beckhove, K.H. Kogel, M. Oostendorp, T. Staub, E. Ward, H. Kessmann, J. Ryals, Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8 (1996) 629-643.

[20] K. Lawton, L. Friedrich, M. Hunt, K. Waymann, T. Delaney, Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J. 10 (1996) 71-82.

[21] L. Friedrich, TP. Lawton , W. Ruess, P. Masner, N. Specker, A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J. 10 (1996) 61-70.

[22] Y. Narusaka, M. Narusaka, T. Horio, H. Ishii, Comparison of local and systemic induction of acquired disease resistance in cucumber plants treated with benzothiadiazoles or salicylic acid. Plant Cell Physiol. 40 (1999) 388-395.

[23] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15 (1962) 473-497.

[24] R.B. Horsch, S. Fry, N.L. Hoffman, D. Eichholtz, S.G. Rogers, R.T. Fraley, A simple and general method for transferring genes into plants. Science 227 (1985) 1229-1231.

[25] C. N. Stewart, Jr., M. J. Adang, J. N. All, P. L. Raymer, S. Ramachandran, W. A. Parrott, Insect control and dosage effects in transgenic canola containing a synthetic *Bacillus thuringiensis crylAc* gene. Plant Physiol. 112 (1996) 115-120.

[26] M. E. Palm, W. Gam, H. Nirenberg, *Plectosporium*, a new genus for *Fusarium tabacinum* anamorph of *Plectosporium cucumerina*. Mycologia 87 (1995) 397-406.

[27] C. N. Stewart, Jr., L. E. Via, A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. BioTechniques 14 (1993) 748-751.

[28] H.A. Richards, M.D. Halfhill, R.J. Millwood, C.N. Stewart, Jr., In review. Quantitative GFP fluorescence as an indicator of recombinant protein synthesis in transgenic plants. Plant Cell Rep.

[29] J. B. Rasmussen, J. A. Smith, S. Williams, W. Burkhart, E. Ward, S.C. Somerville, J. Ryals, R. Hammerschmidt, cDNA cloning and systemic expression of acidic peroxidases associated with systemic acquired resistance to disease in cucumber. Physiol. Mol. Plant Pathol. 46 (1995) 389-400.

[30] D. Mohnen, H. Shinshi, G. Felix, F. Meins, Jr. Hormonal regulation of ß-1, 3-glucanases messenger RNA levels in cultured tobacco tissue. EMBO J. 4 (1985) 1631-1635.

[31] F. Mauch, M. B. Mauch, T. Boller, Antifungal hydrolases in pea tissue:iInhibition of fungal growth by combination of chitinase and ß-glucanase. Plant Physiol. 88 (1988) 936-942.

[32] J. Mantis, B.W. Tague, Comparing the utility of ß-glucuronidase and green fluorescent protein for detection of weak promoter activity in *Arabidopsis thaliana*. Plant Mol. Biol. Rep. 18 (2000) 319-330.

[33] F., Nagy, M.Y., Boutry, M.-Y.,Hsu M., Wong, & N.-H. Chua, The 5'-proximal region of the wheat Cab-1 gene contains a 268-bp enhancer-like sequence for phytochrome response EMBO J. 6 (1987) 2537-2542.

Figure Legends

Fig. 1. RT-PCR of *gn1/gfp* plants. Lane 1 contains DNA marker, lane 2 contains water as a negative control, lane 3 contains nontransgenic plant DNA, lane 4 contains plasmid DNA containing the *mgfp5-er* gene, lane 5 contains cDNA from a *35s-mgfp5-er* transgenic plant, lanes 6-10 contain cDNAs from *gn1/gfp* plants. RNA extraction was performed 7 days after plant induction with 5 mM BTH.

Fig. 2. Western blot analysis of (T_1) transgenic plants induced with BTH. Four and eight week-old plants grown on 3 mM BTH in Magenta boxes (A) and (B) respectively. Twelve-week-old plants grown in soil were sprayed with 5 mM BTH (C). Lane 1 contains a protein marker, lane 2 contains a 20 ng GFP standard. Lane 3 contains positive control plant containing a *35s/mgfp5-er* fusion construct. Lane 4 contains transgenic plant sprayed with water (negative control). Lanes 5-12 are independent *gn1/gfp* transgenic events sprayed with 5 mM BTH. Lanes contain *gn1/gfp* # 1, 2, 3, 4, 5, 6, 8, and 11, which express *gfp*. The samples were collected from the lower leaves of T_1 tobacco plants.

Fig. 3. GFP fluorescence detection by using UV light in roots of a *gn1/gfp* transgenic plant after spraying with BTH. Roots from a transgenic plant containing *35s/mgfp5-er* were used as a positive control, nontransgenic plant roots as negative control (WT), roots from a *gn1/gfp* line sprayed with 1mM and 5 mM BTH. GFP fluorescence was slightly detectable as light green compared to control plants.

Fig. 4. GFP fluorescence detection by using UV light in leaves of a *gn1/gfp* transgenic plant after spraying with BTH. a) Leaves from a *35s/mgfp5-er* transgenic plant used as a positive control, a nontransgenic plant as a negative control (WT), leaves from *gn1/gfp* plants sprayed with 1mM and 5 mM BTH. GFP fluorescence was slightly detectable as light green compared to control plants.

Fig. 5. Systemic induction of *gn1/gfp* gene; western blot analysis of 12 week-old T_1 tobacco plants harboring pBinplus Gn1 sprayed with 5 mM BTH. The samples were taken 4 days after induction. Lane 1 contains a protein marker, lane 2 contains a 20 ng GFP standard. Lane 3 contains a sample from a *35s/mgfp5-er* transgenic plant used as a positive control. Lane 4 contains a sample of a nontransgenic (Xanthi) plant sprayed with BTH. Lane 5 contains a sample from the fourth leaf of a Gn1 plant sprayed with water. Lane 6 represents a *gn1/gfp* plant sprayed with BTH. Transgenic event *gn1/gfp* # 8 was sprayed with 5 mM BTH to determine systemic induction. The leaf samples were collected from the lower leaves of tobacco plants.

Fig. 6. Western blot showing an induction time course of GFP in BTH-sprayed plant tissues. Leaves were sprayed with 5 mM BTH. Lane 1 contains a protein marker, lane 2 contains a 20 ng GFP standard, lane 3 is a sample of a positive control plant transgenic for *35s/mgfp5-er*, lane 4 contains a sample from a *gn1/gfp* transgenic plant not sprayed with BTH, lane 5 contains a *gn1/gfp* transgenic plant sample sprayed with BTH taken at the same day (day 0), lanes 6 -13 contain samples 2, 4, 6, 8, 10, 12, 14, 16 days after

sprayed with BTH. The leaf samples were collected from the lower leaves of a 12 week-old T_1 transgenic event.

Fig. 7. Western blot analysis on T_1 tobacco plants inoculated with pathogen. Leaves were collected 7 days after inoculation. Lane 1 contains a protein marker, lane 2 contains a GFP standard (20 ng), lane 3 contains a positive control sample: *35s/mgfp5-er*, lane 4 contains nontransgenic control plant sample sprayed with water*,* lane 5 contains a sample from a nontransgenic control plant inoculated with *Plectosporium tabacinum*. Lanes 6-12 contain samples from *gn1/gfp* transgenic events # 2, 5, 6, 61, 8, 14, 18 inoculated with *Plectosporium* spores. The samples were collected from the lower leaves close to the inoculated area.
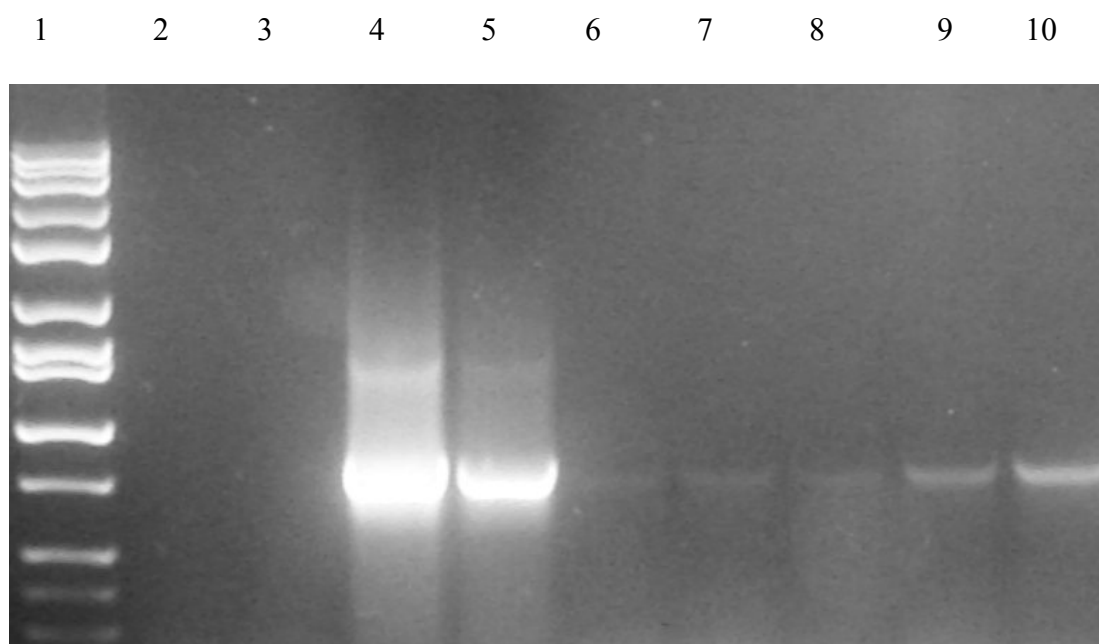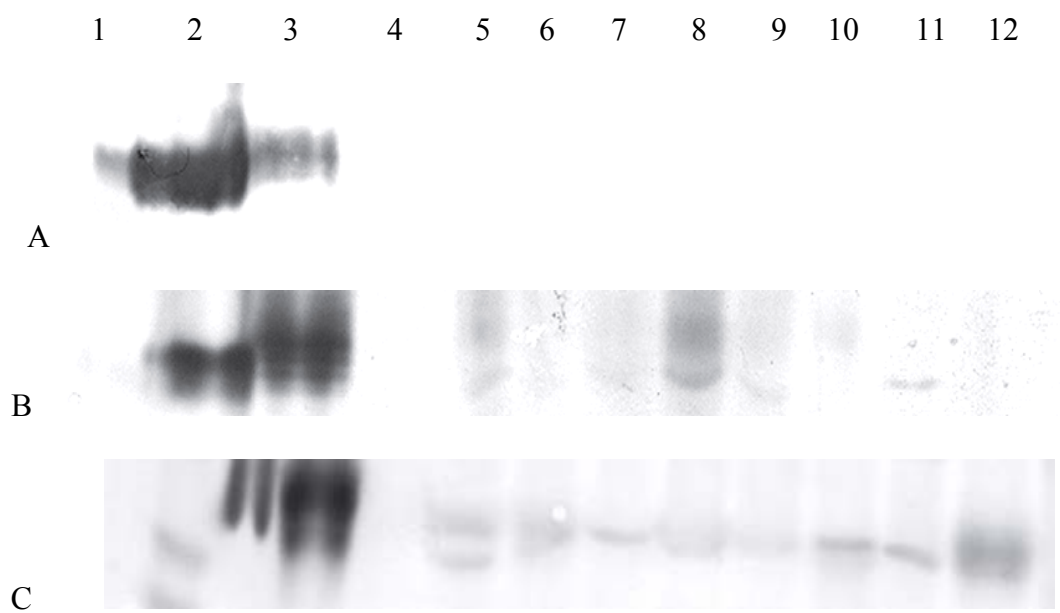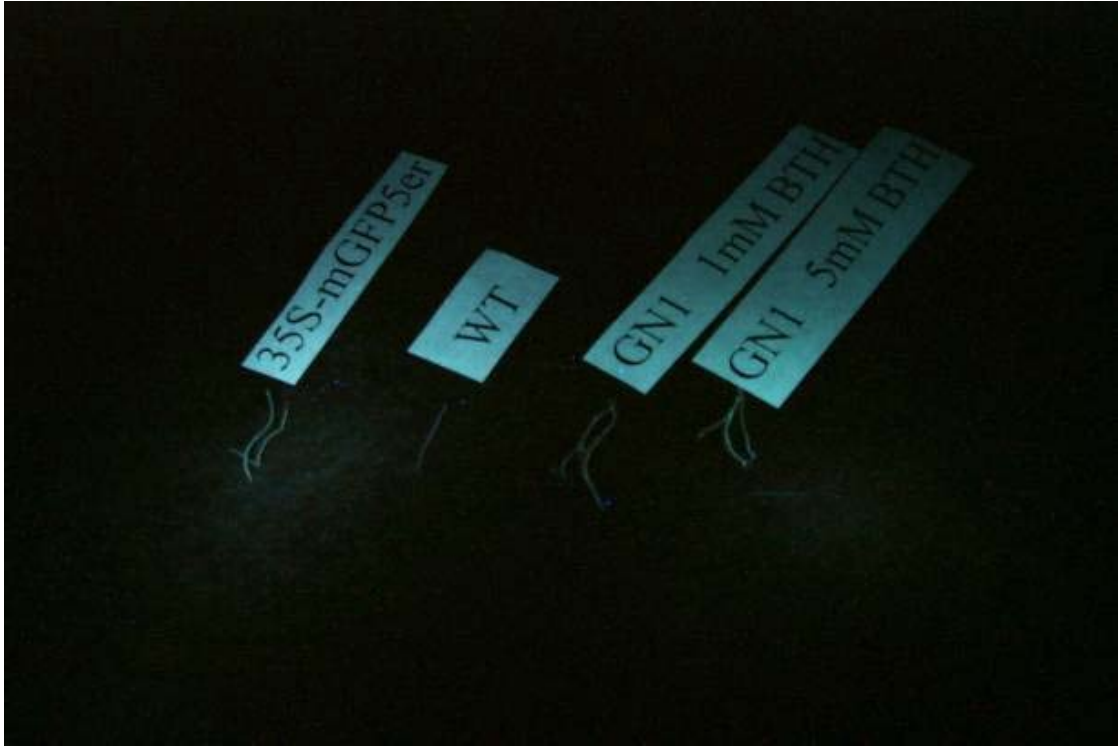
Fig. 1.

1 2 3 4 5 6 7 8 9 10 11 12

A

B

C

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

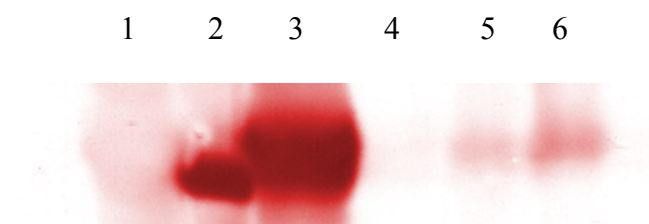| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |

Fig. 6.

1 2 3 4 5 6 7 8 9 10 11 12
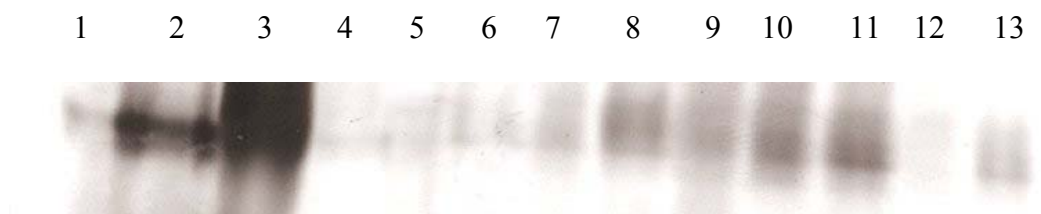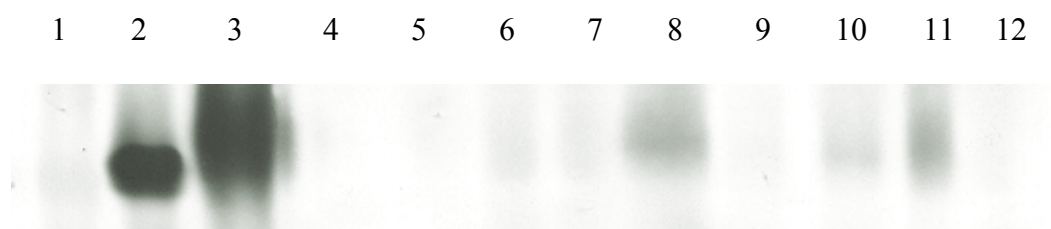
Fig. 7.

Part 2

Radiometric Modeling of GFP-enhanced Plants

One of the critical goals of the GFP efforts at the Special Technologies Laboratory has been to determine the optimum configuration for detecting expression under field conditions. Several data collections have been performed in Santa Barbara as part of this work. In these collections laser-induced fluorescence imaging (LIFI), laser-induced spectroscopy (LIFS), and emission-excitation matrices (EEM's) were used to collect the fluorescence behavior at the "whole-plant" level. Digital photography was also collected. While LIFI and digital photography give the spatial extent of expression and the plants' general appearance, LIFS and EEM's give the spectral content. Work is now focused on modeling to show how different sensors would view the expression. Both ground-based and airborne platforms will be considered. Ultimately, the scenario drives the optimum choice of sensor and platform.

In a semi-diverse plant canopy, there are many fluorescent materials in the field-of-view; from weak emissions from humic soils, to bright emissions from plant structures such as flowers. All fluorescence sources that are not indicative of gene expression must be considered as backgrounds that could interfere with detection. Plants imaged in Santa Barbara were affected by a number of environmental factors such as pathogen attack, and yellowing. These effects caused some spectral changes, but most were limited to changes in the blue emission wavelengths. However, these changes must be considered when using processing some algorithms to enhance detection.

The LIF model has been resurrected for use here on genetically-enhanced plants. The model is not completely intuitive, but good progress has been made in understanding all inputs. The radiometric model was used primarily for detecting man-made materials. Plants often served as the background interferences and these materials will be used in the model if possible. The model is based in TKsolver™, a direct solving program that uses empirical data such as camera noise, fluorescence yields, reflectance spectra with independent variables such as altitude (distance), aperture, laser power, etc. Currently, the model describes five optical geometries of varying distance and camera types (intensified video, PMT, etc.). This is being updated with newer camera/detector specifications.

Quarterly Input (October-December):

This quarter's efforts have been directed at modifications to the existing radiometric model for GFP plants as targets and processing EEM data to produce calibrated spectra for the model. Last quarter, the third data collection was completed. During the three collections in Santa Barbara, EEM matrices, LIFI imagery and LIFS spectra were collected from stems and leaves of Canola plants, and leaves of Tobacco plants. GFP protein concentrations were measured by Dr. Stewart's lab for the third (June FY02) collection on Canola and Tobacco. The data analysis and modeling have focused on the Canola data, and the processing of these spectra has been completed. A limited amount of Tobacco plants will be analyzed if appropriate. Currently, the data processing and

input is user intensive as spread sheets are used to correct both for the additional filters and normalization of spectra to a known fluorescence standards. Of the 25 excitation wavelengths used to excite fluorescence, emission spectra from 355-nm and 390-nm excitations were converted into quantum efficiencies and placed in the model. These excitation wavelengths were chosen because they were the two laser wavelengths were used in LIFI and LIFS collections.

Plant	Control	GFP-12	GFP-2	GFP-2	GFP-9
Canola	5	6	6	-	-
Tobacco	7	4	-	-	6

An intern and an STL staff member have worked through model to become more familiar with TKsolver and updated menus and routines for interpreting plant signatures. One of the results observed from plant imaging experiments was that the fluorescence of the GFP response was not spatially uniform. For the plants investigated in this work, older leaves of always-expressing plants tend to have little or no observed GFP fluorescence, so the target leaf may be surrounded by a number of nearly identical leaves that have no GFP expression. The model addresses this potentially low contrast situation by using the GFP-modified plant spectrum as the signal and an averaged control spectrum as the dominant noise. Another condition to be considered in the model involves spatial resolution. If the imaging resolution of the receiver is less than the feature size (leaf or stem), the signal pixel will contain background emission in the signal pixel. When the pixel size is set to be larger then the leaf size, the canopy signal return is treated as a "mixed pixel".   The signal return will be considered a weighted sum of GFP fluorescence in leaves that are expressing, and other materials including non expressing leaves. To the degree that background fluorescence fills the pixel, contrast is diminished. These model changes are proceeding but have not been completed to date. Next quarter, the model will be run using a relevant scenario for remote detection.